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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12Q 1/68 — A1

(11) International Publication Number: WO 95/24503

(43) International Publication Date: 14 September 1995 (14.09.95)

(21) International Application Number: PCT/EP95/00823

(22) International Filing Date: 6 March 1995 (06.03.95)

(30) Priority Data:
108879 7 March 1994 (07.03.94) IL

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(81) Designated States: AU, CA, CZ, FI, HU, JP, KR, MX, NZ, PL, RO, RU, SK, UA, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: SENSITIVE CANCER TEST

(57) Abstract

An extremely sensitive assay for the early detection of human cancer, of various types, is based on the use of a molecular marker, designated as Gene H19, which is used for *in situ* hybridization of a tissue sample and for indicating the absence or presence of a malignancy and its grading by a suitable marker. The probe can be derived from the H19 gene by subcloning at a suitable site in a plasmid, antisense RNA is produced by transcription with a polymerase and suitable fragments are labelled to produce, after hybridization, the desired signal. The label can be radioactive or fluorescent, or a color reagent can be used. Amongst malignancies assayed are a trophoblastic tumor, bladder carcinoma, ovarian teratoma, pediatric Wilms' Tumor, rhabdomyosarcoma, and testicular cancer. Also, a kit for carrying out such an assay is provided.

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DESCRIPTION

SENSITIVE CANCER TEST

FIELD OF THE INVENTION:

There is provided an extremely sensitive assay for the early detection of human cancer, of various types. The assay is based on the use of a molecular marker, designated as Gene H19, which is used for in-situ hybridization of a tissue sample and for indicating the absence or presence of a cancer and its grading by a suitable marker (probe). Also, a kit for carrying out such an assay is provided.

BACKGROUND OF THE INVENTION:

Despite intensive therapeutic efforts cancer death rates are increasing. In the US they went up by 7 percent between 1975 and 1990. Should this trend continue, by the year 2000 every third individual in the western countries will harbor a potentially fatal malignancy. A major reason accounting for treatment failure is its administration to patients with a biologically advanced disease. A deceivingly small one cubic centimeter of tumor contains about a billion cancer cells. As a rule, patient prognosis - i.e., risk of recurrence and death, is directly correlated with the extent of the disease at the time of diagnosis: the more advanced the disease, the poorer the patient's chances are. Only a strategy leaning heavily on a) prevention and b) early detection could result in substantial gains. The leads indicating the importance of an early diagnosis

come from analysis of US as well as of Japanese statistics. In the former, cancers of the urinary bladder (TCC) and of the uterine cervix and in the latter stomach cancer have shown reduced mortality. These achievements are due to early diagnosis and to screening of populations at risk. Current diagnostic tools, besides physical examination, are mainly imaging by (conventional or CT) X-rays and ultrasonography. Usually, their resolution power can detect lesions only if larger than one cubic cm. Biochemical testing of tumor products released into the blood stream, tumor "markers", is considerably more sensitive. Likewise, specific staining of biopsied tissues can detect minimal disease.

Genomic imprinting - the uniparental transmittance of a genetic trait - plays a pivotal role in embryogenesis and fetal development, and has been linked to tumorigenesis and human disease. H19 is an imprinted gene in human, expressed from the maternal allele. It is extensively transcribed early in embryogensis and in certain fetal tissues, and its expression is shut off in postnatal life. The expression of H19 parallels, in general, the expression of insulin-growth-factor 2, to which it is tightly linked on chromosome 11p15.5. The H19 gene does not encode for a protein and functions on an RNA molecule.

Relaxation of imprinting of H19 has been demonstrated in Wilms' tumor as well as in trophoblastic neoplasia. We have studied the

expression of H19 in several types of human cancer exhibiting features of tissues which expresses H19 in fetal life: transitional cell bladder tumors. Wilms' tumor and rhabdomayosarcoma, as well as gynogenetic germ cell tumors. Two low-grade transitional cell tumors of the urinary bladder did not express the gene, like in bladder mocosa of the adult. Prominent expression of H19 was evident in 3 intermediate-grade and 4 high-grade transitional cell carcinomas and in in-situ bladder carcinoma adjacent to invasive tumor. H19 was found to be expressed in nephrogenic rests in a kidney of aniridia syndrome and in Wilms' tumor, as well as in 4/6 cases of embryonal rhabdomyosarcoma. Expression of H19 was noted in epithelial and mesenchymal elements of immature ovarian teratoma which developed after excision of dermoid cyst, while the original tumor did not express the gene. Prominent expression of this gene was also noted in certain elements of testicular germ cell and stromal-sex cord tumors. Genomic imprinting is a newly discovered mechanism in genetics by which certain traits are selectively expressed either from the maternal or from the paternal genome. Genomic imprinting plays a pivotal role in early stages of embryogensis and implantation as well as in fetal development.

Our understanding of the scope of genomic imprinting is continuously expanding, and imprinted genes have been linked to an array of human diseases. Of major interest is the role of imprinted genes in the evolution of cancer. One of the genes proved to be imprinted in man is the H19 gene. It is located on chromosome 11p15.5, in close proximity to the gene of insulin-like growth factor 2 (IGF2), and is reciprocally imprinted: H19 is expressed from the maternal allele, while IGF2 is expressed from the paternal allele (Bartolomei 92). H19 is one of the most abundant mRNAs in the fetus, comprising up to 10% of the total mRNA in the placenta and certain tissues. The H19 gene does not encode for a protein, and presumably acts as a RNA molecule. In spite of extensive study, its function has remained an enigma. On the basis of loss of heterozygosity at 11p15.5 with retention of the paternal chromosome in Wilms' tumor and embryonal rhabdomyosarcoma and in the cancer-predisposing syndrome of Beckwith-Wiedeman. It has been suggested that H19 should be considered a candidate for tumor suppressor gene. Recently, it has been shown that H19 is expressed from the maternal as well as the paternal alleles in about a third of Wilms' tumors. Shortly thereafter expression of H19 has been demonstrated in complete hydatidiform mole, which is placental tissue exclusively derived from androgenetic genome, and that gives rise to a malignant trophoblastic tumor - choriocarcinoma. This phenomenon of the aberrant expression of H19 from the paternal allele has been coined relaxation of imprinting.

SUMMARY OF THE INVENTION:

There is provided an extremely sensitive assay for the early detection of human cancer, of various types. The assay is based on the use of a molecular marker, designated as Gene H19, which is used for in-situ hybridization of a tissue sample and for indicating the absence or presence of a cancer and its grading by a suitable marker (probe). Also provided a kit for carrying out such assay. As set out above, there has been identified a molecular marker, a gene designated as H19, located on chromosome 11 in the vicinity of several cancer related genes. According to the invention, an assay is provided, based on in-situ hybridization (ISH) technique to analyze tissue sections for H19 expression.

We identified a molecular marker, a gene designated H19, located on chromosome 11 at a vicinity to several cancer related genes. By in-situ hybridization (ISH) technique applied to tissue section, the probe can tell whether the tissue is H19 positive or negative.

We have studied, so far, the following malignancies: trophoblastic tumors, ovarian teratomas, Wilms' tumor, rhabodomyosarcome (a muscle tumor), carcinoma of the urinary bladder and testicular germ-cell and sex cord stromal tumors. In the bladder carcinoma expression was positively correlated with tumor grading and malignant potential. Additional common cancers, among which are breast, colon, lung, pancras, skin, kidney and prostate cancers, are currently being studied for H19 expression.

To investigate the association of H19 expression with neoplasia we examined its expression as demonstrated by in-situ hybridization in several human tumors and preneoplastic conditions. We have chosen tumors which share morphologic features and tissue-specific markers with tissues which express H19 in fetal life, especially those tumors known to be linked to chromosomal aberrations on the short arm of chromosome 11 in the malignant or premalignant state. We also looked at H19 expression in gynogenetic ovarian mature and immature teratomas.

TRANSITIONAL CELL BLADDER TUMORS:

The transitional cell tumors, arising from the epithelium lining the urinary collecting system, is one of the most prevalent types of cancer in the human.

The histopathologic grading of transitional cell neoplasma is based on their resemblance to the normal tissue and correlates well with their biologic behavior, including the potential to become invasive.

Prominent expression of H19 was found in the transitional epithelium of the renal pelvis and ureter and of the urinary bladder of the fetus but not in the bladder mucosa of the adult.

No expression of H19 was detected in two cases of low grade well differentiated papillary (grade 1 out of 3) bladder carcinoma. Prominent expression was found in 3 intermediate grade (2) (one

with submucosal invasion and one with muscularis invasion) and 4 cases of high grade (3) invasive bladder carcinoma.

Pronounced expression of H19 was also noted in carcinoma in-situ of the bladder mucosa adjacent to invasive cancer. These findings suggest that expression of H19 correlates with the stage of differentiation and hence with the invasive potential of transitional cell bladder carcinoma, and possibly can serve as a marker in cases of low to intermediate malignancy to indicate a more aggressive behavior.

Wilms' Tumor:

Wilms' tumor is a pediatric tumor of the kidney which resembles the fetal kidney and contains elements of the primitive metanephric blastema differentiating to tubular and/or glomerular structures admixed with mesenchymal stroma. Wilms' tumor is associated with several hereditary disorders, among which are Beckwith-Wiedemann syndrome, hemihypertrophy and the aniridia syndrome.

The expression of H19 in the fetal kidney is prominent in the primitive metanephric blastema and is dramatically reduced with differentiation to tubular structures. In a kidney removed from a patient with aniridia syndrome. H19 expression was demonstrated in rests of metanephric blastema, while no expression was detected in the rest of the mature renal tissue. Expression of H19 was evident in Wilms' tumor, as already described by others.

RHABDOMYOSARCOMA:

Embryonal rhabdomyosarcoma is a solid pediatric tumor with morphologic and biochemical markers of striated muscle at early stages of differentiation. During embryogenesis pronounced H19 expression is found in primitive mesenchyme and myoblasts of the developing muscular system of the human embryo, but the expression is markedly reduced with differentiation of myotubes. With formation of myofibers in the fetus in the second trimester of pregnancy H19 expression is again abundant with residual low expression postnatally.

H19 expression was examined to 6 cases of embryonal rhabdomyosarcoma. The histologic diagnosis in all cases routinely included positive histochemical staining for muscle markers and/or electron microscopic study. H19 was found to be expressed in 4/6 embryonal rhabdomyosarcoma.

OVARIAN TERATOMA:

Two cases of immature ovarian teratoma following surgical excision of dermoid cyst (mature cystic teratoma) were examined for expression of H19, as well as one of the primary benign tumors. H19 was found to be expressed in mesodermal and endormal elements in immature teratoma, but not in the preceding dermoid cyst compared to mature tissue elements.

TESTICULAR GERM-CELL AND SEX-CORD-STROMAL TUMORS:

Prominent expression of H19 was found in certain components non-seminomatous germ cell tumor, but no expression was disclosed in seminomas.

Expression of H19 was also found in stromal-sec-cord testicular tumors.

METHODS:

1. Preparation of H19 probe for in-situ hybridization: A part of the human H19 gene (800bp) was subcloned onto a plasmid. In-vitro transcription with T7 RNA polymerase was used to produce antisense H19 RNA from linearized plasmid DNA using [^{35}S] UTP nucleotide, and was purified by ethanolic precipitation.

2. In-Situ Hybridization: Paraffin blocks were sectioned at 5 microns onto TESPA (Sigma) coated microscope slides. Sections were dewaxed with xylene, fixed by O.2M paraformaidehyde solution and treated with proteinase K. The next steps were treatment with acetic acid anhybride and dehydration. Hybridization was performed using [^{35}S] RNA probes according to Standard procedures. The unhybridized probe was removed after 12h of hybridization. The slides were exposed to a photographic emulsion for 4-6 days, developed using Kodak D19 developer and fixed with Kodak fixer. The slides were counterstained using Hematoxylin-eosin, examined and photographed using a light microscope under bright and dark field illumination. This method may be utilized for examination of histological and cytological slides from all tissues. To facilitate rapid laboratory examination the H19 antisense probe

will be labeled with a fluorescentic tag or color which will shorten exposure tune to 24h.

Fluorescentic labelling of another oncodevelopmental gene (IGF2) by a dye which emits fluorescent light at a different wavelength will allow visualization of both gene products on the same slide. Analysis of these slides requires only a fluorescence microcope. Some newly available commercial kits for in-situ hybridization combined with fluorolabelled HI9 and IGF2 probes will comprise a user-friendly laboratory package.

CLAIMS:

1. An assay for indicating the presence or absence of certain malignancies and their grading, which comprises in-situ hybridization of a tissue of the patient to be examined, with a probe derived from the H19 gene, and evaluating the results obtained.

2. An assay according to claim 1, where the probe is derived from the H19 gene by subcloning at a suitable site in a plasmid, antisense RNA is produced by transcription with a polymerase and suitable fragments are labelled to produce, after hybridization, the desired signal.

3. An assay according to claim 2, where the label is a radioactive or fluorescent, or where a color reagent is used.

4. An assay according to any of claims 1 to 3, where the malignancy assayed is a trophoblastic tumor, bladder carcinoma, ovarian teratoma, pediatric Wilms' Tumor, rhabdomysarcoma carcinoma of the bladder, or testicular cancer.

5. An assay according to any of claims 1 to 3 to analyze and use H19 as a tumor marker in any other type of human cancer.

6. An assay according to any of claims 1 to 3 to analyze and use a tumor marker in cytologic specimens.

7. An assay according to any of the claims 1 to 6 where IGF2 is used as marker for human cancer in histologic and cytologic preparations.

8. A kit for carrying out an assay according to claims 1 to 7 by radioactive or fluorescent or color reaction of H19 and/or IGF2 probes.

9. A kit for carrying out an assay according to claims 1-7, which comprises fluorescent H19 and IFG2 probes emitting at different wave lengths.

10. A kit for carrying out an assay as claimed in any of claims 1 to 7, substantially as herein described.

INTERNATIONAL SEARCH REPORT

Intern: al Application No
PCT/EP 95/00823

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	INT. J. ONCOL., (1993) 2/5 (753-758), DOUC-RASY ET AL. 'Expression of the human fetal BAC/ H19 gene in invasive cancers' see the whole document ---	1-10
X	AM J HUM GENET, (1993 JUL) 53 (1) 113-24, ZHANG ET AL. 'Imprinting of human H19: allele-specific CpG methylation, loss of the active allele in Wilms tumor, and potential for somatic allele switching. ' see the whole document ---	1-10
X	NAT GENET, (1993 JUN) 4 (2) 110-3., FEINBERG 'Genomic imprinting and gene activation in cancer' see the whole document ---	1-10
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[X] Further documents are listed in the continuation of box C. [ ] Patent family members are listed in annex.

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14 July 1995	19.07.95

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	NATURE, (1993 APR 22) 362 (6422) 747-9., RAINIER S; JOHNSON L A; DOBRY C J; PING A J; GRUNDY P E; FEINBERG A 'Relaxation of imprinted genes in human cancer. ' ---	
A	AM. J. HUM. GENET., (1993) 53/5 (1096-1102), MUTTER ET AL. 'Oppositely imprinted genes H19 and insulin-like growth factor 2 are coexpressed in human androgenetic trophoblast.' ---	
A	CELL GROWTH DIFFER, (1993 DEC) 4 (12) 1013-21, OWEN ET AL. 'Coordinate regulation of collagen II(alpha 1) and H19 expression in immortalized hamster cells' ---	
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